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600-1-294N

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT : Lee, Sung et al.
SERIAL NO. : 10/677,097 EXAMINER : Hanley, Susan Marie
FILED : October 1, 2003 ART UNIT : 1651
FOR: GLYCOSYLATED LPXTGases AND USES THEREOF

CERTIFICATE OF MAILING UNDER 37 CFR 1.8

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(Name of Person Depositing Mail)

Loretta Kavanagh 12/11/2006
(Signature and Date)

DECLARATION UNDER 37 C.F.R. §1.132

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SIR:

I, VINCENT FISCHETTI, hereby declare and state that:

1. I am a Professor and Head of the Laboratory of Bacterial Pathogenesis and Immunology at Rockefeller University having received my Ph.D. degree from New York University in 1970. After that I was a postdoctoral fellow at Rockefeller University And the Albert Einstein College of Medicine. My full curriculum vitae is attached hereto as EXHIBIT A.

2. My principal area of research is Bacterial Pathogenesis specializing in Gram-Positive bacteria, and among other positions I serve as reviewer in numerous funding agencies, including the NSF, NIH, DARPA. I also have served as reviewer for numerous

scientific journals including J. Experimental Medicine, Infection and immunity, J. Bacteriology, Molecular Microbiology. I have also served as Editor-in-Chief of Infection and Immunity for 10 years.

3. In the course of my activities, I have been listed as inventor on several patent applications, including the one noted above entitled "GLYCOSYLATED LPXTGases AND USES THEREOF", having U.S. Serial Number 10/677,097, filed October 1, 2003, which claims priority to U.S. provisional application Serial Number 60/415,273, filed October 1, 2002.

4. I have reviewed the present application, including subject matter that may be directly applied to the isolation of a new enzyme, which we refer to as LPXTGase, which cleave proteins in, or at a site C terminal to, an LPXTG motif found in cell surface proteins of gram positive bacteria. More particularly, at the time of filing the patent application noted above, we had identified an LPXTGase enzyme from both *Streptococcus pyogenes* and *Staphylococcus aureus*, both of which cleaved a peptide fragment obtained from the cell surface M protein of *Streptococcus* (a virulence factor) that contains this LPXTG motif. While the application contains the data directed to the studies with *S. pyogenes*, we clearly state on page 10, paragraph [0042] of the application that:

> "A similar enzyme, found in the membrane extract of *Staphylococcus aureus,* suggests that this unusual molecule may be common among gram positive bacteria."

5. Thus, at the time of filing the above-noted patent application, we had determined that this enzyme isolated from *Staphylococcus aureus* had similar properties and activity as the *Streptococcus pyogenes* enzyme, in particular, it had the same molecular mass (14,000 daltons) as that of the enzyme isolated from *Streptococcus pyogenes*, it was glycosylated like the enzyme from *Streptococcus pyogenes* and it demonstrated the same specificity for cleaving proteins that contained an LPXTG motif. Support for this is presented in the paper attached here as EXHIBIT B (Lee, SG et al. (2002), J. Biol. Chem. 277, No. 49,

pp.46912-46922), which is the paper on which the present application was based. See in particular on page 46921, left column, in the middle of the last paragraph, whereby we state:

> *"This has been verified from our recent finding that S. aureus also produces an enzyme strikingly similar to the LPXTGase of S. pyogenes. The S. aureus enzyme cleaves the LPXTG motif, has a similar molecular weight, and is glycosylated like the S. pyogenes enzyme."*

While we had not published this data at the time of filing the application, the Materials and Methods and results obtained from these earlier studies on the isolation of the enzyme from *Staphylococcus*, which were done at the time of filing the present application, are attached here as EXHIBIT C.

6. To further prove that the enzymes were the same, we then prepared an antibody to the *S. pyogenes* enzyme, and performed studies to see if it cross-reacted with the enzyme isolated from *Staphylococcus aureus*. The results of this analysis show that this antibody recognized both the *Streptococcus*, as well as the *Staphylococcus* enzyme, thus providing conclusive evidence that the enzymes are the same. The results of this study are also shown in EXHIBIT C.

7. Additional studies were done to prove that the enzyme is present in other species of *Streptococci*. We have performed the same isolation protocols and analyses on *Streptococcus pneumoniae* and have determined that an enzyme with the same physical and biochemical characteristics, as well as, protein cleavage specificity, has been identified in *S. pneumoniae*

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18 of

the U.S. Code, Section 1001, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Dated: 12/6/06

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Education:
Ph.D. New York University, Microbiology, 1970, (with honors)
M.S. Long Island University, Microbiology, 1967
B.S. Wagner College, Bacteriology, 1962

Training and Experience:

1990 -Pres.	Professor and Chairman, Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, New York, N.Y.
1978 - 1990	Associate Professor, The Rockefeller University, New York, N.Y.
1973 - 1978	Assistant Professor, The Rockefeller University, New York, N.Y.
1970 - 1979	Adjunct Assistant Professor, Adelphi University, Garden City, N.Y.
1972 - 1973	Guest Investigator, The Rockefeller University, New York, N.Y.
1972 - 1973	Postdoctoral Fellow, Albert Einstein College of Medicine, N.Y.
1970 - 1972	Postdoctoral Fellow, The Rockefeller University, New York, N.Y.

Honors and Awards (selected):

2006	Loyd Harris Lecturer, U. of Oklahoma
2006	G.F. Heinrich keynote lecture, Lang Center, NY Hospital
2006	Division M Keynote Address, ASM
2006	Chair, NY Academy of Sciences, Microbiology Division
2005	COBRE Visiting Scholar, U. Hawaii
2004	Keynote address, Southern California ASM
2004	McLaghlan Lecturer, University of Texas, Galveston
2004	ASM Lecturer, American Society of Virology, Montreal
2003	Ellison Medical Foundation Lecture (Wind River Conference, CO)
2003	"State of the Art Lecture", Am. Society of Virology, Davis, CA
2003	Chair, Gordon Conference, Chemical and Biological Terrorism Defense
2003	Invited speaker, ASM Biodefense Conference, Baltimore, MD
2002	Invited speaker, Banbury meeting on Bacteriophage Biology, CSH, NY
2002	Invited speaker, Boston University, Boston, MA
2002	Cover article - Nature, Aug. 22. "Defense against Anthrax"
2002	Keynote address, International Organization for Mycoplasmology
2001	Guest speaker, Swiss Society of Intensive Medicine
2000	Keynote address, Joint German Conference for Microbiology
1999	Keynote address, Japanese Lancefield Society Annual Meeting
1999	John H. Hanks Memorial Lecture, Johns Hopkins School of Public Health
1999	Pfizer Lectureship (U. Pittsburgh)

1997 - 2007	**MERIT Award**, National Institutes of Health
1996 - 1997	Foundation Lecturer, American Academy for Microbiology
1996	Keynote address, International Lancefield Society, Paris
1995	Invited speaker, Institut Pasteur Symposia, "The Year of Louis Pasteur"
1994 - Pres	Fellow, American Academy for Microbiology
1994 - 1995	Chairman, Div. B (Microbial Pathogenesis), Am. Society for Microbiology
1992	McLaghlan Lecturer, University of Texas, Galveston
1992	Burroughs Wellcome Visiting Professor, (University of Arizona)
1987 - 1997	**MERIT Award**, National Institutes of Health
1987	Invited speaker, Institut Pasteur Centennial
1987	Shipley Lecturer, Harvard Medical School
1986 - 1987	President, Lancefield Society
1980	Alumni Achievement Award, Wagner College
1977 - 1982	Research Career Development Award, National Institutes of Health
1973 - 1977	Senior Investigator, New York Heart Association
1971 - 1973	Helen Hay Whitney Foundation Fellowship
1970	NYU Founders Day Award for outstanding scholarship

Professional Activities (selected):

2004 - Pres	Scientific Advisory Board, Great Lakes Regional Center of Excellence
2002- Pres	Chief Scientific Advisor, Enzybiotics, LLC
1999 - Pres	Microbiology Advisory Board, New York Academy of Sciences
1994 - Pres	Board of Scientific Advisors and Trustee, Trudeau Institute
1992 - Pres	Advisory Editor, Trends in Microbiology
1990 - Pres	Advisory Editor, Journal of Experimental Medicine
1986 - Pres	Advisory Board, New York Hall of Science
2000 - 2003	Awards Advisory Board, American Society for Microbiology
1996 - 2002	Scientific Advisory Board, SIGA Technologies
1999 - 2002	Chairman, Institutional Review Board (IRB), Rockefeller University
1996 - 2000	Advisory Board, Defense Advanced Research Projects Agency (DARPA)
1996 - 2001	Chief Scientific Advisor, SIGA Pharmaceuticals
1989 - 1999	**Editor-in-Chief,** Infection and Immunity
1984 - 1998	Co-Director, Biotechnology Facility at the Rockefeller University
1995 - 1997	Member, Scientific Advisory Board, Spectral Diagnostics
1993 - 1995	Chairman, Scientific Advisory Board, M6 Pharmaceuticals
1988 - 1989	Assistant Editor, Journal of Experimental Medicine
1980 - 1985	Section Editor, Journal of Immunology
1978 - 1983	NIAID Bacteriology and Mycology Study Section (BM2)
1978 - 1989	Editorial Board, Infection and Immunity
1978 - 1980	Editorial Board, Journal of Immunology

Patents:	US Patent No.
1. Production of streptococcal M protein immunogens	# 4,784,948
2. Streptococcal immunoglobulin A binding protein	# 5,352,588
3. Immunoglobulin binding protein ML2.2	# 5,556,944
4. Regulation of exoproteins in *Staphylococcus aureus*	# 5,587,288
5. Method for exposing group A streptococcal antigens and an improved diagnostic test for the identification of group A streptococci	# 5,604,109
6. Polypeptide of a hybrid surface protein by bacteria	# 5,616,686
7. Process, apparatus and reagents for isolating cellular components (Commercialized as "Fast-prep" RNA/DNA isolation system by Q-biogene)	# 5,634,767

8. Gene for serum opacity factor	# 5,707,822
9. Delivery and expression of a hybrid surface protein by bacteria	# 5,786,205
10. Use of Gram-positive bacteria to express recombinant proteins	# 5,821,088
11. Production of streptococcal M protein	# 5,840,314
12. Fibronectin/fibrinogen binding protein of group A streptococci	# 5,910,441
13. Method for screening inhibitors of the enzyme which cleaves the anchor of surface proteins from Gram-positive bacteria	# 5,968,763
14. Regulation of exoprotein in Staphylococcus aureus II	# 5,976,792
15. Prophylactic and therapeutic treatment of group A streptococcal infections	# 5,985,271
16. Recombinant poxvirus and streptococcal M protein vaccine	# 5,985,654
17. Therapeutic treatment of group A streptococcal infections	# 5,997,862
18. Therapeutic treatment of group A streptococcal infections	# 6,017,528
19. Topical treatment of streptococcal infections	# 6,056,955
20. Use of bacterial phage associated lysing enzymes for the prophylactic and therapeutic treatment of various illnesses	# 6,056,954
21. Plasmin binding protein and therapeutic use thereof	# 6,190,659
22. Use of bacterial phage associated lysing enzymes for treating various illnesses	# 6,238,661
23. Bacterial phage associated lysing enzymes for treating dermatological infections	# 6,248,324
24. Use of phage associated lytic enzymes for treating bacterial infections of the digestive tract	# 6,254,866
25. Parenteral use of bacterial phage associated lysing enzymes for the therapeutic treatment of bacterial infections	# 6,264,945
26. Composition incorporating bacterial phage associated lysing enzymes for treating dermatological infections	# 6,277,399
27. Use of bacterial phage associated lysing enzymes for treating streptococcal infections of the upper respiratory tract	# 6,326,002
28. Receptor for Mycobacterium leprae and methods of use thereof	# 6,331,405
29. Use of bacterial phage associated lysing enzymes for treating bacterial infections of the mouth and teeth	# 6,335,012
30. Receptor for *Mycobacterium leprae* and methods of use thereof	# 6,331,405
31. Fibronectin/fibrinogen binding protein of group A streptococci	# 6,355,477
32. Composition for treatment of a bacterial infection of the digestive tract	# 6,399,097
33. Composition for treating dental caries caused by *S. mutans*	# 6,399,098
34. Composition for treatment of ocular bacterial infection	# 6,406,692
35. Composition for treatment of a bacterial infection of upper respiratory tract	# 6,423,299
36. Vaginal suppository for treating group B streptococcal infections	# 6,428,784
37. Use of bacterial phage associated lysing enzymes for treating dermatological infections	# 6,432,444
38. Synthetic peptides from streptococcal M protein and vaccines prepared therefrom	# 6,602,907
39. C1 bacteriophage lytic system	# 6,608,187
40. Chewing gum containing phage associated lytic enzymes for treating streptococcal A infections	# 6,685,937
41. Method for the treatment of bacterial eye infections	# 6,875,431
42. Tampon for the treatment of streptococcal group B infections	# 6,881,403
43. Therapeutic treatment of upper respiratory infections using a nasal spray	# 6,893,635
44. Method of treatment of vaginal infections	# 6,899,874
45. Nasal spray for treating streptococcal infections	# 7,014,850
46. Syrup composition containing phage associated lytic enzymes	# 7,063,837

47. Use of bacterial phage associated lysing enzymes for treating upper respiratory illness	#7,141,241

Societies: American Society for Microbiology, Kunkel Society, New York Academy of Sciences

BIBLIOGRAPHY
Vincent A. Fischetti, Ph.D.

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ORIGINAL ARTICLES:

1. **Fischetti, V.A., B. Barron, and J.B. Zabriskie.** 1968. Studies on streptococcal bacteriophage. I. Burst size and intracellular growth of group A and group C streptococcal bacteriophages. **J. Exp. Med. 127**:475.

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3. **Barron, B., V.A. Fischetti, and J.B. Zabriskie**. 1970. Studies of bacteriophage kinetics in multicellular systems: a statistical model for the estimation of burst size per cell in streptococci. **J. Applied Bact. 33**:436.

4. **Ellis, R.J., C.W. Lillehei, V.A. Fischetti, and J.B. Zabriskie**. 1970. Heart-reactive antibody: a measure of heart rejection in human heart transplants. **Circulation, XLI and XLII**, Supplement II. 81-97.

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9. **Fischetti, V.A., E.C. Gotschlich, G. Siviglia, and J.B. Zabriskie.** 1976. Streptococcal M-proteins extracted by non-ionic detergent. I. Properties of the antiphagocytic and type-specific molecules. **J. Exp. Med. 144**:32.

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11. **Fischetti, V.A.** 1977. Streptococcal M protein extracted by non-ionic detergent. II. Analysis of the antibody response to the multiple antigenic determinants of the M protein molecule. **J. Exp. Med. 146**:1108.

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13. **Fischetti, V.A.** 1978. Streptococcal M protein extracted by non-ionic detergent. III. Correlation between immunological cross-reactions and structural similarities with implications for antiphagocytosis. **J. Exp. Med. 47**:1771.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 277, No. 49, Issue of December 6, pp. 46912–46922, 2002
Printed in U.S.A.

Characterization of a Unique Glycosylated Anchor Endopeptidase That Cleaves the LPXTG Sequence Motif of Cell Surface Proteins of Gram-positive Bacteria*

Received for publication, August 23, 2002, and in revised form, October 2, 2002
Published, JBC Papers in Press, October 4, 2002, DOI 10.1074/jbc.M208660200

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The precursors of most surface proteins on Gram-positive bacteria have a C-terminal hydrophobic domain and charged tail, preceded by a conserved LPXTG motif that signals the anchoring process. This motif is the substrate for an enzyme, termed sortase, which has transpeptidation activity resulting in the cleavage of the LPXTG sequence and ultimate attachment of the protein to the peptidoglycan. While screening a group A streptococcal membrane extract for cleavage activity of the LPXTG motif, we identified an enzyme (which we term "LPXTGase") that differs significantly from sortase but also cleaves this motif. The enzyme is heavily glycosylated, which is required for its activity. Amino acid composition and sequence analysis revealed that LPXTGase differs from other enzymes, in that the molecule, which is about 14 kDa in size, has no aromatic amino acids, is rich in alanine, and is 30% composed of uncommon amino acids, suggesting a nonribosomal construction. A similar enzyme found in the membrane extract of *Staphylococcus aureus*, indicates that this unusual molecule may be common among Gram-positive bacteria. Whereas peptide antibiotics have been reported from bacillus species that also contain unusual amino acids and are synthesized non-ribosomally on amino acid-activating polyenzyme templates, this would be the first reported enzyme that may be similarly synthesized.

A large group of cell surface proteins of Gram-positive bacteria are covalently anchored through their C termini to the cell wall peptidoglycan. Most of these proteins are essential for pathogenic bacteria to establish successful infection of host tissues, and hence they are considered virulence factors. Functionally, these surface proteins may be divided into three major groups: *viz.* 1) those with adhesin or invasion function (1–27); 2) those with antiphagocytic activities (28–40); and 3) those that are enzymes that degrade surface components of host cells, thereby facilitating spread, and enzymes that hydrolyze large molecules in the surroundings into utilizable nutrients (29, 41–48).

A striking feature of all of these functionally and structurally diverse surface proteins is that they all possess a carboxyl-terminal LPXTG sequence (49), which is cleaved during surface translocation at the septum (50), resulting in a covalent linkage to cell wall peptidoglycan. In all cases, the genes for these proteins contain additional nucleotide sequences following that which encodes the LPXTG. These additional sequences encode a stretch of hydrophobic amino acids and positively charged C-terminal amino acids. Pancholi and Fischetti (51) observed that the hydrophobic and positively charged amino acid sequences are missing in the cell wall-linked M protein, indicating that the precursor of M protein was cleaved at a site within or immediately C-terminal to the LPXTG sequence. These findings strongly indicated that surface proteins become anchored to the cell wall by a common mechanism (49). Subsequently, it was shown that deletion of either the LPXTG or hydrophobic amino acid sequence or charged terminal amino acid from the precursor of protein A of *S. aureus* results in failure of protein A anchoring to the cell wall (52), indicating that these sequences were essential for the cell wall-anchoring process of these proteins. Collectively, these sequences are considered to be a cell wall sorting signal, which has now been shown to be present in over 100 surface proteins of Gram-positive bacteria (53, 54).

Through a series of elegant experiments, Schneewind *et al.* (55) have shown that the peptide bond between threonine and glycine of the LPXTG sequence of protein A becomes cleaved by an enzyme termed sortase, after which the carboxyl terminus of threonine becomes covalently attached to the amino group of one of the glycines of the pentaglycine cross-bridge of the *S. aureus* cell wall. Recently, they have shown that *S. aureus* mutants defective in the anchoring of surface proteins to the cell wall carry a mutation in *srt* gene (50). Subsequently, they cloned the *srtA* gene in *Escherichia coli* and purified recombinant sortase. *In vitro*, the purified sortase cleaved the LPXTG sequence after threonine (56) and also covalently attached the surface protein with C-terminal LPXT to a triglycine substrate (57). These results indicate that sortase possesses two functions, a specific endopeptidase and a transpeptidase. In addition, they showed that *S. aureus* mutants lacking sortase are unable to display surface proteins and are defective in establishing infection (58). An analysis of the genome of several Gram-positive bacteria revealed that there is more than one sortase gene per bacterial genome (54).

In the present report, we have identified and purified an enzyme from *Streptococcus pyogenes* that actively cleaves the LPXTG anchor motif but is very different from the sortase of *S. aureus* in its glycosylation and presence of uncommon amino acids. We here describe the physical and biochemical properties of this enzyme, which we term LPXTGase.

EXPERIMENTAL PROCEDURES

Enzymes—*N*-Glycosidase F (catalog no. 1,365,193) and *O*-glycosidase (catalog no. 1,347,101) were purchased from Roche Molecular Biochemicals. β-*N*-Acetylhexosaminidase (A7708) and β-glucosidase (G6906)

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* This work was supported in part by a grant from SIGA Technologies and United States Public Health Service Grant AI11822 (to V. A. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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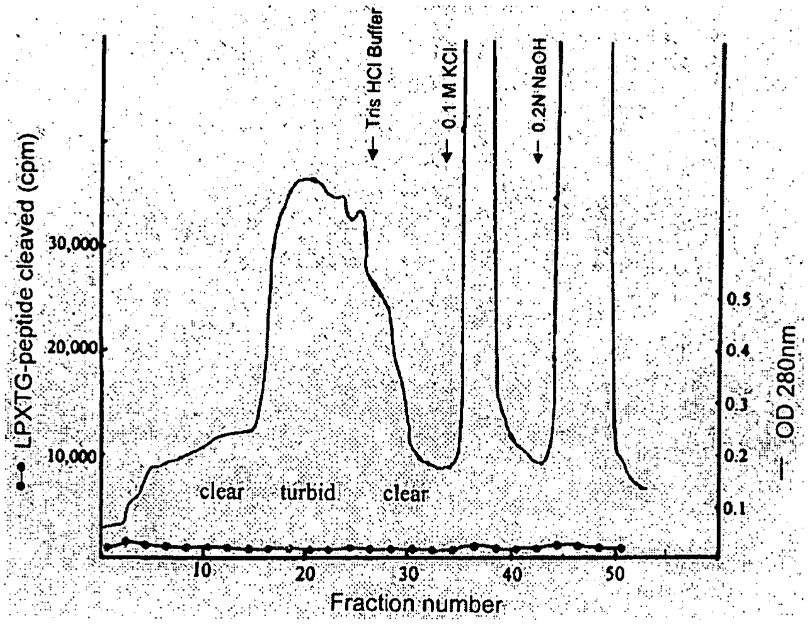

FIG. 1. **DEAE-cellulose chromatography of the cytosol fraction.** The cytosol fraction containing membrane vesicles was chromatographed on a DEAE-cellulose column as described under "Experimental Procedures." Aliquots (10 μl) of fractions were assayed for cleavage activity of bead-bound ^{125}I-labeled LPXTG-containing peptide as described under "Experimental Procedures." The solution eluting from the column in the fractions appeared clear and turbid.

were purchased from Sigma. Group C streptococcal C1 phage lysin was prepared by the method described by Nelson *et al.* (59).

Other Materials—Silica gel TLC plates (catalog no. M 5729-6), solvents, and other chemicals were purchased from Fisher. Carboxymethyl glass beads (G-3910) and 1-ethyl-3-(dimethylaminopropyl) carbodiimide (E-1769) and buffers were purchased from Sigma. ^{125}I was purchased from PerkinElmer Life Sciences.

Bacterial Strain and Culture—*S. pyogenes* strain D471 was grown in 50 liters of Todd-Hewitt medium supplemented with 1% yeast extract in a fermenter. Cells were harvested when the OD at 650 nm reached 1.0. To harvest the cells, the culture was concentrated to about 2 liters by means of a Millipore Corp. Procon filtration apparatus, and the concentrated culture was centrifuged for 10 min at 8,000 rpm using a GSA rotor. Cell pellets (about 120 g total wet weight) were suspended in 1.5 liters of 30 mM MES[1] buffer, pH 6.2, and the cells were pelleted again by centrifugation.

Cell Lysis and Preparation of Crude Extract—The washed cell pellets described above were suspended in 1.2 liters of 30 mM MES buffer, pH 6.2, and cell clumps were gently dispersed with a Dounce homogenizer. To the cell suspension 10,000 units of lysin, a muralytic enzyme of a group C phage origin (60), were added, and the mixture was stirred for 1 h at 37 °C. Treatment with a low concentration of lysin resulted in localized digestion of cell wall peptidoglycan, creating holes in the cell wall. Cell membrane and cytosol exuded through these holes, releasing cytosol and part of the cell membrane as vesicles (59, 61). The cell ghosts were pelleted by centrifugation for 20 min at 10,000 rpm with a GSA rotor, and the supernatant containing cytosol and membrane vesicles was collected. The cell ghosts were then resuspended in 600 ml of the MES buffer, the suspension was centrifuged again, and the supernatant was collected. The combined supernatant, termed the cytosol fraction, was used as a starting material for enzyme purification. The cell ghosts, which retained the remainder of the cell membrane, were suspended in 800 ml of the MES buffer, and after adding Brij 35 to a final concentration of 0.2%, the mixture was stirred overnight at 4 °C. The mixture was then centrifuged for 30 min at 10,000 rpm, and the resulting supernatant, termed the membrane extract, was also used as a starting material for enzyme preparation.

Preparation of LPXTG Peptide Substrate—An LPXTG-containing peptide, KRQLPSTGETANPFY from the streptococcal M6 protein, was synthesized by the Rockefeller University Peptide Synthesis Laboratory. The C-terminal tyrosine was added to allow the peptide to be labeled with ^{125}I using IODOBEADs. Generally, 2 mg of the peptide was labeled with 1 mCi of ^{125}I. The N terminus of the labeled peptide was then linked to carboxymethyl glass beads by carbodiimide catalysis according to the manufacturer's instructions. To achieve the linkage, the peptide (1.2 μmol) was incubated for 7 h at 37 °C with 200 mg of the glass beads (59 μmol of carboxyl termini) and 30 μmol of 1-ethyl-3-(dimethylaminopropyl) carbodiimide with gentle shaking in 2 ml of 100 mM MES buffer, pH 4.9. The reaction mixture was placed in a small column, and unreacted peptide was removed by washing the column with 300 ml of 1 M Tris-HCl buffer, pH 8.6, containing 1% SDS, and then with 1 liter of distilled water. Similar bead-bound peptides in which the LPSTGE was reversed (EGTSPL) or randomly placed (TEPGSL) were synthesized, labeled, and purified in the same way.

Purification of LPXTGase—To about 1.8 liters of the cytosol fraction, Brij 35 was added to a final concentration of 0.1%, and the fraction was applied to a DEAE-cellulose column (16 × 4.3 cm) equilibrated with 20 mM Tris-HCl buffer, pH 6.8. About 500 ml of clear solution absorbing UV at 280 nm eluted first, followed by turbid UV-absorbing solution. After all of the applied cytosol fraction entered the column, the column was washed with 20 mM Tris-HCl buffer, pH 7.6, containing 0.1% Brij 35. The wash step eluted additional turbid solution and then eluted a clear UV-absorbing solution. Generally, 500 ml of wash buffer was required until UV absorbance returned to base line. The column was then eluted with 0.1 M KCl in 20 mM Tris-HCl buffer, pH 7.6, and 0.1% Brij 35 and finally with 0.1 N NaOH. The clear fall-through eluant was collected and saved while the turbid fall-through eluant was reapplied to a second DEAE-cellulose column of similar dimensions, and the clear eluant was collected. The turbid eluant that followed was applied to a third DEAE-cellulose column, and the column was eluted in the same manner. The pool of clear eluants amounting to about 3 liters was concentrated to 30 ml using an Amicon ultrafiltration apparatus fitted with a YM3 membrane with a 3-kDa molecular mass cut limit. One-half of the concentrated solution was applied to a Sephadex G50 column (60 × 4.3 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.6, containing 0.1% Brij 35; the column was then eluted with the same buffer solution, and 20-ml fractions were collected.

Enzyme Assay Method—Aliquots (10 μl) were removed from the column fractions and added to 1.5-ml Microfuge tubes. To each tube was added 30 μl of 40 mM Tris-HCl buffer, pH 7.6, containing 0.1% Brij 35, followed by a 10-μl suspension of bead-bound LPXTG peptide substrate (0.5–0.8 μg, 100,000–200,000 cpm). The reaction mixture was incubated with vigorous shaking for 60 min at 37 °C. After this time, 100 μl of water was added to each tube, and after vortex mixing, the mixture was centrifuged at 10,000 rpm for 5 min to pellet the beads. 100 μl of the supernatant was withdrawn, and radioactivity was counted with a γ counter.

Optimization of Enzyme Activity—To determine the pH optimum of enzyme activity, enzyme reactions were carried out under various pH conditions using the following buffers: MES-NaOH (pH 5, 6, 6.5, and 7), Tris-HCl (pH 7.5, 8.0, 8.5, and 9), NH_4OH-HCl (pH 10), and triethylamine HCl (pH 11 and 12). To determine the effect of detergents on enzyme activity, enzyme reactions were carried out in the presence of 0.05–0.5% of Brij 35 or Triton X-100. To determine the optimal duration

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[1] The abbreviations used are: MES, 4-morpholineethanesulfonic acid; HPLC, high pressure liquid chromatography; PTH, phenylthiohydantoin.

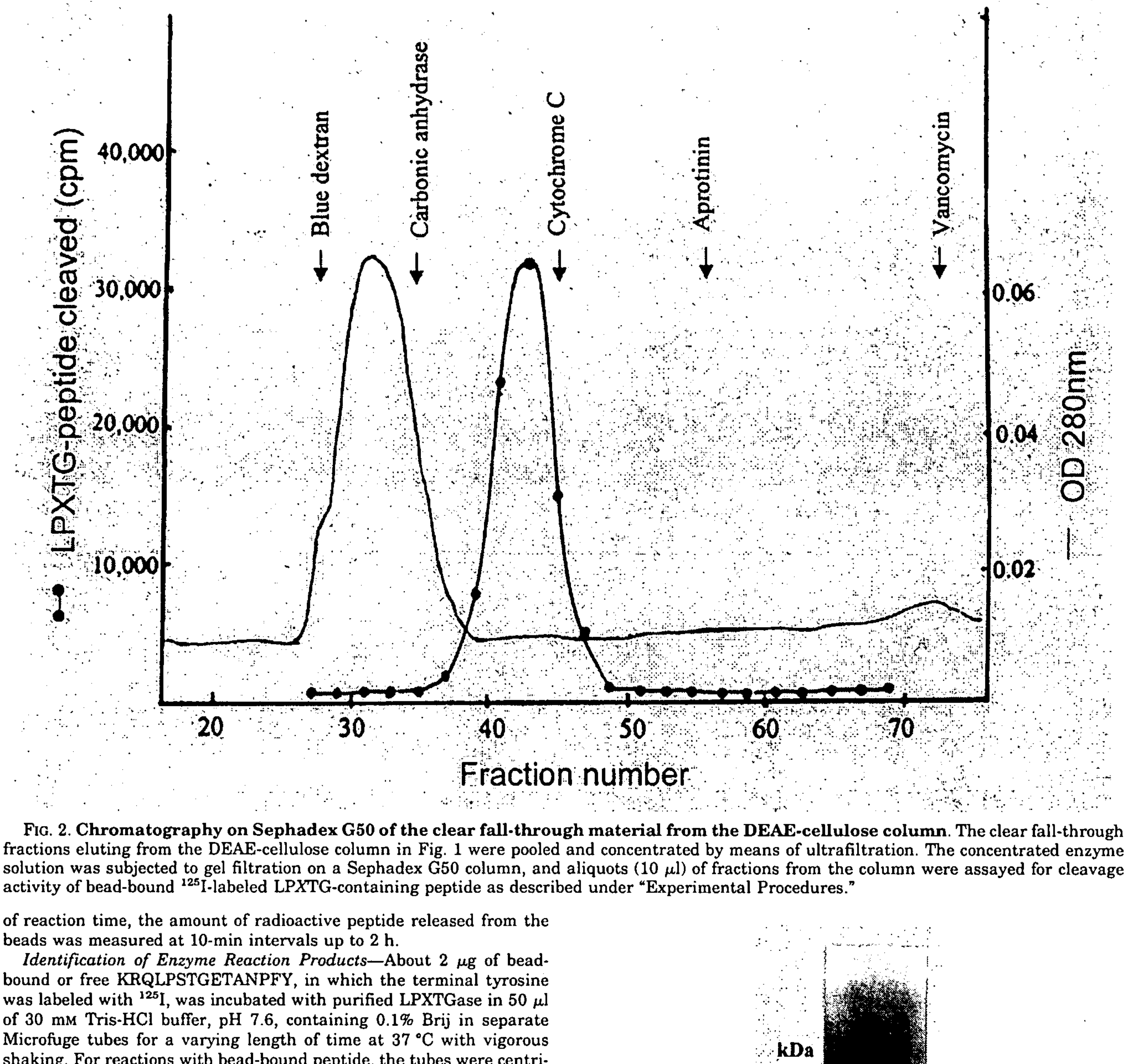

FIG. 2. **Chromatography on Sephadex G50 of the clear fall-through material from the DEAE-cellulose column.** The clear fall-through fractions eluting from the DEAE-cellulose column in Fig. 1 were pooled and concentrated by means of ultrafiltration. The concentrated enzyme solution was subjected to gel filtration on a Sephadex G50 column, and aliquots (10 μl) of fractions from the column were assayed for cleavage activity of bead-bound ^{125}I-labeled LPXTG-containing peptide as described under "Experimental Procedures."

of reaction time, the amount of radioactive peptide released from the beads was measured at 10-min intervals up to 2 h.

Identification of Enzyme Reaction Products—About 2 μg of bead-bound or free KRQLPSTGETANPFY, in which the terminal tyrosine was labeled with ^{125}I, was incubated with purified LPXTGase in 50 μl of 30 mM Tris-HCl buffer, pH 7.6, containing 0.1% Brij in separate Microfuge tubes for a varying length of time at 37 °C with vigorous shaking. For reactions with bead-bound peptide, the tubes were centrifuged at designated times in order to pellet unreacted bead-bound peptide, and 30-μl aliquots of the supernatant were spotted onto a silica gel TLC plate. For reaction with free peptide, 30-μl aliquots from each tube at designated times were directly spotted on a silica gel TLC plate. The plates were developed with a solvent mixture consisting of ethyl acetate/pyridine/acetic acid/water (60:30:9:24), and the reaction products were located by autoradiography. Reaction products were eluted from the plate, and amino acid sequences were determined with an Applied Biosystems AB1 Procise 494 instrument by the Rockefeller University protein chemistry laboratory.

Various concentrations of LPXTGase or trypsin (as control) was added to 50 μg of bovine serum albumin (U. S. Biochemical Corp.) under conditions described above and incubated for 1 h at 37 °C. A sample of the reaction mixtures was analyzed by SDS-PAGE for degradation.

Enzyme Kinetics—Varying concentrations of ^{125}I-labeled KRQLPSTGETANPFY peptide, ranging from 20 to 240 μM were incubated with 2.4 μM of the purified enzyme in 50 μl of 50 mM Tris-HCl buffer, pH 7.6, containing 0.1% Brij 35 at 37 °C for 30 min. At the end of the reaction time, 30 μl of the reaction mixtures were spotted on silica gel TLC plates, and the plates were developed with ethyl acetate/pyridine/acetic acid/water (60:30:9:24). The plates were then autoradiographed, and substrate and reaction products were scraped off the plate and counted for radioactivity.

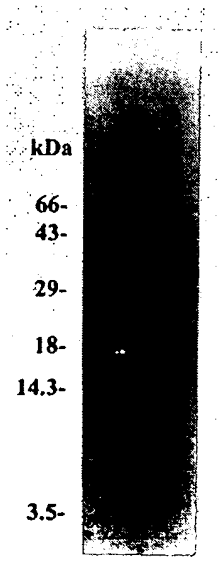

FIG. 3. **SDS-PAGE analysis of the ^{125}I-labeled LPXTGase.** The ethanol and ethyl acetate-precipitated enzyme that had been labeled with ^{125}I-tyramine was solubilized in 0.2% SDS in 100 mM Tris-HCl buffer, pH 6.8, and after boiling, the enzyme was subjected to SDS-PAGE using 16% acrylamide gel, and the dried gel was autoradiographed.

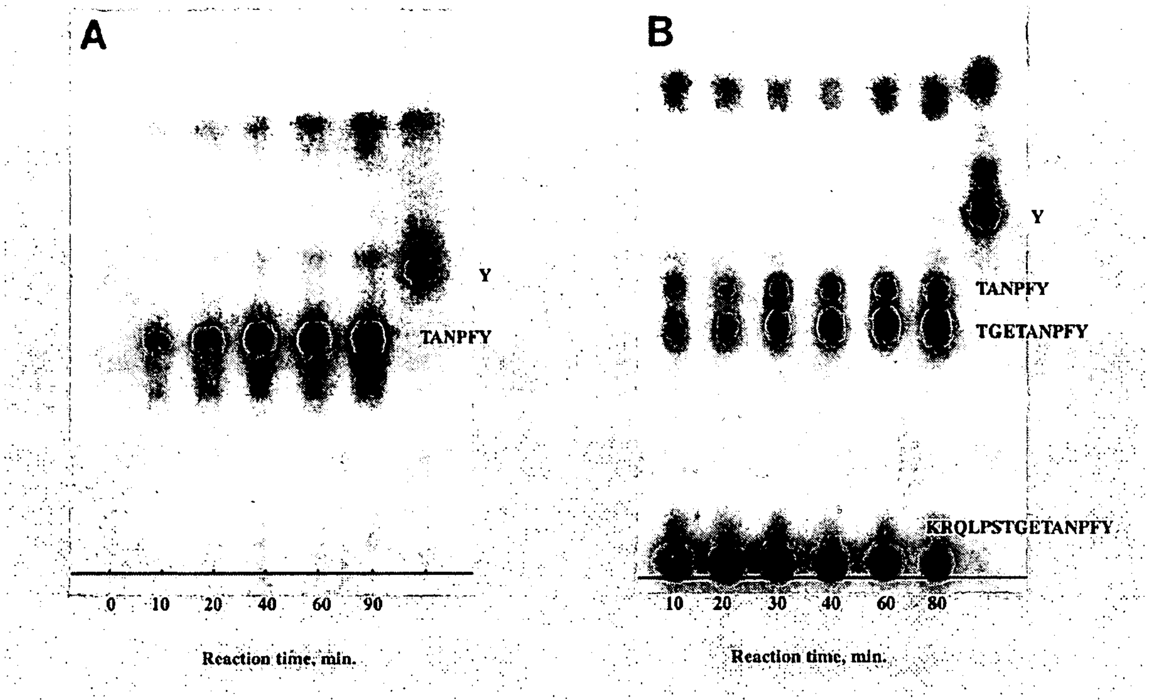

FIG. 4. **Cleavage of an LPXTG peptide by LPXTGase.** *A*, bead-bound KRQLPSTGETANPFY, in which the C-terminal tyrosine was labeled with ^{125}I, was incubated with purified LPXTGase, and the reaction product was examined on silica gel TLC as described under "Experimental Procedures." *B*, free KRQLPSTGETANPFY, in which the C-terminal tyrosine was labeled with ^{125}I, was incubated with purified LPXTGase, and the reaction products were examined on silica gel TLC as described under "Experimental Procedures." *Y*, the location of free tyrosine. The locations of other peptide sequences were identified after sequencing the peptide within their respective spots.

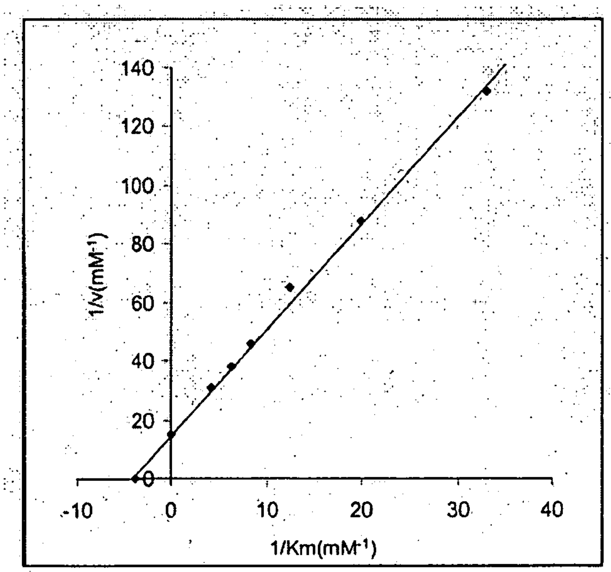

FIG. 5. **The kinetics of cleavage of LPXTG peptide by LPXTGase.** ^{125}I-Labeled KRQLPSTGETANPFY, ranging from 20 to 240 μM, was incubated for 30 min with 2.4 μM LPXTGase, and the kinetics of cleavage of the peptide was determined as described under "Experimental Procedures."

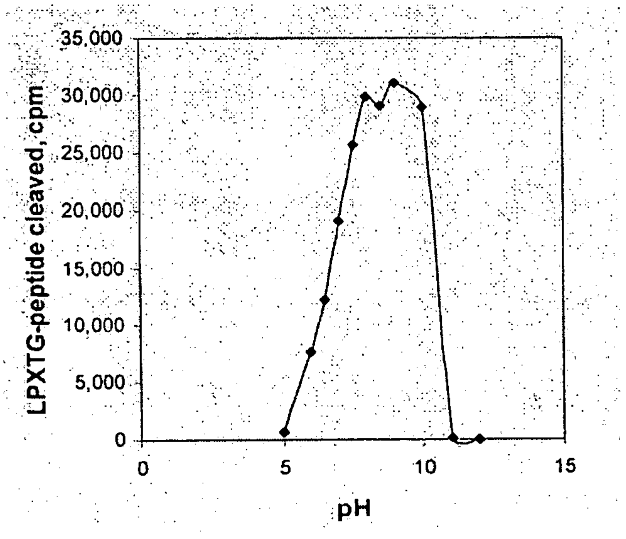

FIG. 6. **pH optimum of LPXTGase activity.** The optimum pH for LPXTGase activity was determined, at 50 mM concentrations of various buffers. The buffers employed were as follows: MES-NaOH for pH 5, 5.5, 6, 6.5, and 7; Tris-HCl for pH 7.5, 8, 8.5, and 9; NH_4OH-HCl for pH 10; and triethylamine HCl for pH 11 and 12.

Enzyme Concentration and Dry Weight Determination—The fractions with enzyme activity eluting from the Sephadex G50 column were combined, and the enzyme solution, amounting to about 150 ml, was concentrated to about 10 ml by YM3 ultrafiltration, after which an aliquot of 2–3 ml was lyophilized. The lyophilized enzyme was dissolved in 200 μl of distilled water, and the concentrated enzyme solution was divided into two preweighed Microfuge tubes. To each tube 300 μl each of ethanol and ethyl acetate was added. The tubes were kept at −20 °C, precipitating the enzyme, leaving most of the Brij 35 and buffer salts in the supernatant. The precipitated enzyme was pelleted by centrifugation, and the pellets were dissolved in a small volume of distilled water. This enzyme solution was used as the starting material for various chemical analyses. For dry weight determination, the precipitate was dissolved in 200 μl of distilled water and the ethanol/ethyl acetate

TABLE I

Effect of salts on LPXTGase activity

Reaction mixtures contained, in 50 μl of 40 mM Tris-HCl, pH 7.6, and 0.1% Brij 35, a fixed amount of LPXTGase, ^{125}I-labeled LPXTG-peptide (about 180,000 cpm), and the indicated concentrations of various salts. The reaction mixtures were incubated for 1 h at 37 °C, and the peptide cleavage was determined as described under "Experimental Procedures."

Salts	Concentration	Peptide cleaved	Percentage of control
	mM	*cpm*	*%*
Sodium chloride	100	6,720	16
Potassium chloride	100	8,608	20
Hydroxylamine HCl	100	11,200	27
Magnesium chloride	20	8,615	20
Calcium chloride	20	9,706	23
Putrescene chloride	20	8,710	21
Sodium phosphate	20	20,646	49
Sodium sulfate	20	23,454	57
EDTA	20	2,147	5.2
Control (no salt)		41,530	100

precipitation step was repeated two more times in order to remove residual detergent and salts, the final precipitate was dried *in vacuo*, and the tubes were weighed.

Amino Acid and Sugar Composition of LPXTGase—The amino acid and sugar composition of the LPXTGase were determined by the Rockefeller University Protein Chemistry Laboratory using a Waters 490 HPLC system. The concentrated enzyme in distilled water described above was used as starting material for these analyses.

Determination of the Amino Acid Sequence of a Tryptic Fragment of the Enzyme—To remove covalently bound carbohydrate from the protein backbone of the LPXTGase, 1.2 mg of the enzyme was incubated for 36 h at 37 °C with vigorous shaking with 10 units of *N*-glycosidase F and 10 milliunits of *O*-glycosidase in 600 μl of 30 mM Hepes buffer, pH 7.6, containing 0.1% Brij 35 in a Microfuge tube. The incubation mixture was then banded on silica gel plates, and the plates were developed twice with 80% ethanol. The deglycosylated protein band was visualized by exposing the plate to iodine vapor. The protein band was eluted with 80% ethanol and concentrated to about 200 μl in a Speedvac (Savant). To the concentrated protein solution, 400 μl of 30 mM Hepes buffer, pH 7.6, containing 0.1% Brij 35, and 5 μg of trypsin were added, and the mixture was incubated overnight at 37 °C. To prepare phenylthiohydantoin (PTH)-labeled tryptic fragments, 3 μl of phenylisothiocyanate, and 300 μl of pyridine were added to the trypsinized enzyme, after which a small volume of 1 N NaOH was added to raise the pH to 9, and the mixture was shaken at 37 °C for 6 h. The reaction mixture was then banded on a silica gel plate and developed with a solvent mixture consisting of *n*-butyl alcohol, hexane, acetic acid, and water (40:40:9:1).

UV-absorbing, PTH-labeled tryptic fragments were located, and the fragments were eluted with 95% ethanol. The slow moving fragment was then subjected to Edman degradation as follows. After hydrolysis of the PTH-peptide by a 30-min exposure to 30% trifluoroacetic acid at 50 °C, the PTH-derivative and residual peptide were separated on a silica gel TLC plate using the same running solvent. The PTH-derivative was located under a UV light, and the residual peptide was located by exposure of the plate to iodine vapor. In this manner, seven PTH-derivatives were prepared. The Rockefeller University Protein Chemistry Laboratory identified the PTH-derivatives. Of the seven PTH-derivatives, four exhibited unusual masses. To help identify them, these derivatives were acid-hydrolyzed, and the Protein Chemistry Service Laboratory again analyzed the resulting products.

Inactivation of LPXTGase by Glycosidases—LPXTGase was preincubated with glycosidases in 40 μl of 30 mM Tris-HCl buffer, pH 7.6, containing 0.1% Brij 35, for 1 h at 37 °C, after which a 10-μl suspension of bead-bound, ^{125}I-labeled KRQLPSTGETANPFY peptide was added, and the reaction mixture was incubated for 1 h. At the end of the incubation, peptide cleavage was determined as described above.

Radiolabeling the LPXTGase—To label the LPXTGase directly for visualization on SDS gels, ^{125}I-tyramine was linked to the enzyme. Tyramine (1 μmol) was incubated for 5 h at 37 °C with 20 μCi of ^{125}I in the presence of IODOBEADs in 200 μl of 20 mM phosphate buffer, pH 6.5. 30 μl of the ^{125}I-labeled tyramine solution was transferred to a Microfuge tube, to which were added about 60 μg of concentrated LPXTGase and 50 μg of 1-ethyl-3-(dimethylaminopropyl) carbodiimide, both in 50 μl of distilled water, and then 20 μl of 1 M MES buffer, pH 4.9, and 2 μl of 10% Brij 35 were added. Finally, distilled water was added to bring the volume of the incubation mixture to 200 μl, and the mixture was incubated for 7 h at 37 °C with gentle shaking. At the end of the incubation, the volume of the incubation mixture was reduced to 50 μl by means of a Speedvac, and the enzyme was precipitated with ethanol and ethyl acetate as described above.

RESULTS

Purification of the LPXTGase—Using the bead-bound ^{125}I-labeled KRQLPSTGETANPFY peptide from *S. pyogenes*, which contains the LPXTG motif, we attempted to identify an endopeptidase in the streptococcal cell extract. In numerous initial trials, no LPXTG cleavage activity was detected in any crude fraction or in fractions from chromatography columns. Eventually, we discovered that ultrafiltrates of the crude extract using a YM3 filter removed a low molecular weight substance that inhibited the cleavage activity. After removing this inhibitor by ultrafiltration, active enzyme could be prepared from both cytosol and membrane extracts. The enzyme activity in the cytosol is entirely attributable to membrane vesicles released into the cytosol rather than a soluble cytosol fraction. For example, lysin treatment of *S. pyogenes* results in localized digestion of the cell wall, producing holes in the cell wall. Through these holes, segments of cell membrane exude externally, which become pinched off as vesicles (59). When the turbid cytosol was centrifuged for 1 h at 30,000 rpm, membrane vesicles along with all of the enzyme activity were pelleted. A large part of the cell membrane still remained associated with the cell ghosts, and more cleavage activity could be extracted from the ghost-associated membranes.

Preliminary experiments revealed that LPXTG cleavage activity does not bind to DEAE-cellulose, whereas most proteins did. Thus, the cytosol fraction containing membrane vesicles or the membrane extract of the cell ghost was directly applied to a DEAE-cellulose column as the first step of enzyme preparation. Free enzyme released from the membrane eluted first in the clear fall-through fraction, which was followed by the vesicle-containing turbid fraction. However, neither the clear nor turbid fall-through fractions showed an LPXTG cleavage activity initially (Fig. 1). Thus, when the volume of the clear fall-through fraction was reduced by ultrafiltration using a 3-kDa cut-off YM3 membrane, the retentate exhibited enzyme activity. This suggests that a low molecular weight inhibitor eluted from the DEAE column together with the enzyme in the fall-through fraction, and the inhibitor passed through the membrane during ultrafiltration. As the concentration of Brij 35 in the fall-through fraction increased during concentration, it formed micelles, which could not pass through YM3 membrane, and as a consequence the retentate became very viscous. Therefore, to remove Brij 35, a large volume of detergent-free Tris-HCl buffer was added to the retentate, and the ultrafiltration process was continued. During this procedure, more of the enzyme inhibitor passed through the YM3 membrane along with monomeric Brij 35, increasing the activity of the enzyme in the retentate.

When the concentrated enzyme solution was subjected to gel filtration using Sephadex G50, an active enzyme peak eluted soon after the void volume (Fig. 2). The activity peak did not absorb UV at 280 nm, indicating that the enzyme does not contain aromatic amino acids. Comparison with the elution profiles of proteins of known molecular weights indicates that the apparent molecular weight of the cleavage enzyme is about 14,000.

SDS-PAGE analysis revealed no protein bands stained with either Coomassie Blue or silver, even when over 100 μg of the purified enzyme was applied to a 16% gel (not shown). However, the lack of protein bands verified the purity of the enzyme preparation. Thus, in order to detect the enzyme in polyacrylamide gels, ^{125}I-labeled tyramine was linked to the carboxyl groups of the enzyme by means of carbodiimide catalysis, and the radioactive enzyme was subjected to SDS-PAGE. Autoradiography of dried gels showed that most of the labeled enzyme was retained in the stacking gel, and only a small amount entered the running gel (Fig. 3). When the enzyme was labeled with fluorescein isothiocyanate and subjected to SDS-PAGE, most of the fluorescent enzyme was located in the stacking gel, with a small amount entering the running gel as a series of faint bands, forming a ladder (not shown) with no fluorescent material at the 14-kDa region. These observations strongly suggest that the enzyme forms large aggregates in the presence of SDS.

Enzyme Reaction Products—To determine where in the LPXTG motif cleavage occurred, purified endopeptidase was used to cleave a bead-bound form of the KRQLPSTGETANPFY peptide, and the cleaved fragment was subjected to N-terminal sequence analysis. Results revealed that the enzyme cleaved after glutamic acid within the LPSTGE sequence, releasing the TANPFY peptide fragment (Fig. 4*A*). On the other hand, the enzyme cleaved the free KRQLPSTGETANPFY peptide at two sites, after the serine and the glutamic acid of LPSTGE, yielding TGETANPFY and TANPFY, respectively (Fig. 4*B*). The fact that only these products were observed indicates that the trypsin (KR) and chymotrypsin (FY) substrates found at either end of the peptide were not cleaved by the endopeptidase. When the enzyme was reacted with similar bead-bound peptides in which the LPSTGE was reversed (EGTSPL) or randomly placed (TEPGSL), no cleavage was observed (not shown). No cleavage was also observed when native bovine serum albumin was reacted in a similar way. The small amount of radioactive materials observed at the solvent front originated from the impurities in the synthetic peptide. These impurities were not reactive to enzyme action. Based on its ability to specifically cleave within the LPXTG anchor motif of surface proteins, we have termed this endopeptidase "LPXTGase."

Enzyme Kinetics—Since the enzyme does not contain any aromatic amino acid, neither the Lowry nor Bradford methods could be used to determine protein concentration. Thus, the enzyme concentration was determined on the basis of its estimated molecular mass (14 kDa) and dry weight. Using this, a Lineweaver-Burk plot of the kinetics of the LPXTGase was determined (Fig. 5), resulting in a K_m of 0.26 mM and a $V_{\max}$ of 67 μM in 30 min when 2.4 μM enzyme was used in the assay.

Optimal Conditions for Enzyme Activity—As shown in Fig. 6, the LPXTGase exhibited a broad pH optimum between 7.5 and 10. Below pH 5 or above 10, the enzyme activity could not be measured. In most enzyme assays, pH 7.6 was used, because background activity was higher at higher pH. Enzyme activity was highest when 0.1–0.2% of both Brij 35 and Triton X-100 was incorporated. The maximal initial enzyme velocity was maintained up to the first 20 min, and after 1 h only small additional cleavage of the peptide occurred.

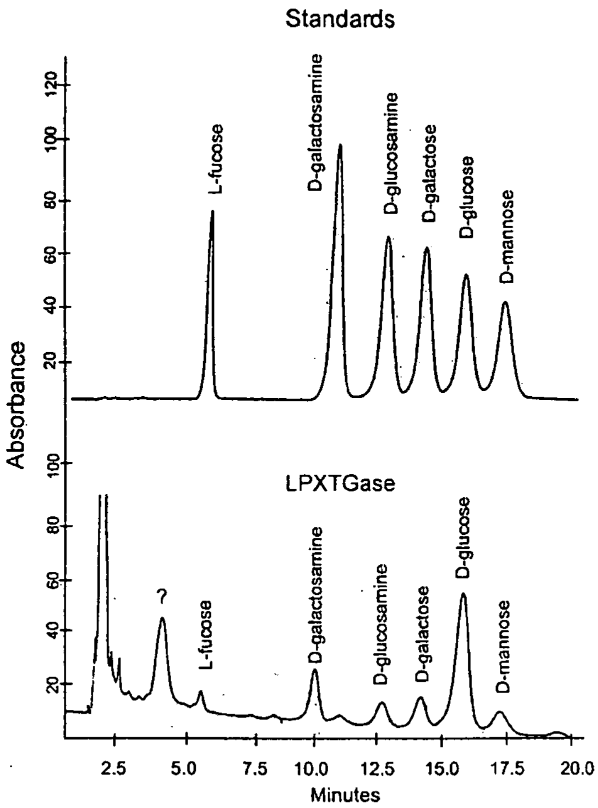

FIG. 7. **Carbohydrate composition of LPXTGase.** The carbohydrate composition of purified LPXTGase was determined by the Rockefeller University Analytical Service Laboratory using a Waters 490 HPLC system.

Inhibition of Activity by Salts—The LPXTGase was exposed to a variety of salts, and its activity was tested for cleavage of the LPXTG-containing peptide. As seen in Table I, the enzyme was found to be rather sensitive to exposure to a number of certain salts.

Presence of Carbohydrate in the LPXTGase—Successful linking of ^{125}I-labeled tyramine to the enzyme demonstrated that it possesses free carboxyl groups. Nonetheless, the enzyme did not bind to DEAE-cellulose, indicating that these carboxyl groups are not surface-exposed. It seemed plausible that the charged amino acids of the enzyme are internalized and that the hydrophobic amino acids are located on the exterior surface in view of the fact that the enzyme is likely to be associated with the cell membrane. However, an enzyme with a hydrophobic surface would not be very soluble in aqueous buffer, which is contrary to our findings. This suggested to us that a few residues of sugars, which would prevent surface exposure of the carboxyl groups but would confer surface hydrophilicity to the enzyme, might shield the carboxyl groups, allowing them to remain soluble in aqueous buffer. To test this, the enzyme was incubated with 5 mM of periodate in 20 mM phosphate buffer, pH 6, for 4 h at 4 °C, and enzyme activity was measured. We found that this treatment nearly completely abolished the enzyme activity, whereas periodate treatment of trypsin in an identical manner showed no effect on its enzyme activity.

When the sugar composition of the LPXTGase was analyzed, we found L-fucose, D-galactose, D-galactosamine, D-glucose, D-glucosamine, and D-mannose in a molar ratio of 1:2:3:13:2:2 (Fig. 7). The aggregate mass of the oligosaccharide is 3,936

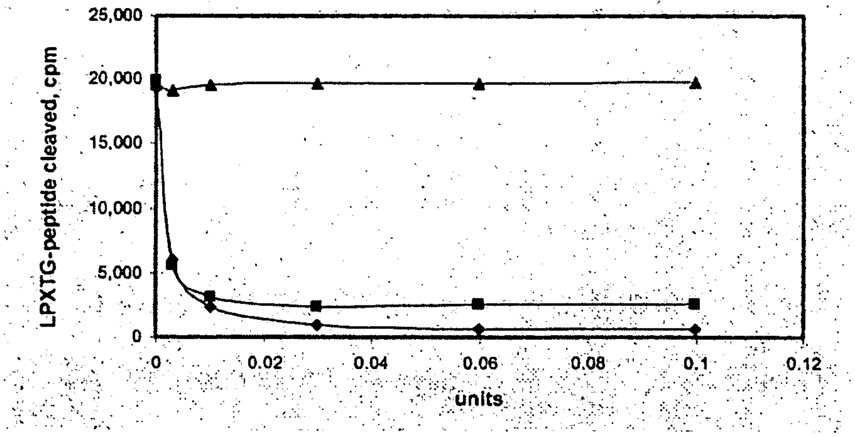

FIG. 8. **Inactivation of LPXTGase activity by glycosidases.** A fixed amount of LPXTGase was preincubated for 1 h with varying amounts of glycosidases at 37 °C in a 40-μl reaction volume of 30 mM Tris-HCl, pH 7.6, containing 0.1% Brij 35. At this time, 10 μl of ^{125}I-labeled, bead-bound LPXTG peptide substrate (about 180,000 cpm) was added, the mixture was incubated at 37 °C for an additional 1 h, and the radioactivity of the cleaved peptide fragment was determined. *Triangle*, β-galactosidase; *square*, *N*-acetylhexosaminidase; *diamond*, β-glucosidase.

daltons. In addition, we found an unidentified sugar that had the shortest retention time from the analytical column. The mass of this unknown sugar residue is estimated to be about 1 kDa. To determine the carbohydrate linkage that is necessary for activity, the LPXTGase was preincubated with a number of glycosidases, prior to testing for endopeptidase activity. As shown in Fig. 8, *N*-acetylhexosaminidase, β-glucosidase, and β-mannosidase abolished the LPXTGase activity, but β-galactosidase had no effect. In addition, *N*-glycosidase F, which cleaves the bond between asparagine and oligosaccharides, abolished the endopeptidase activity, but *O*-glycosidase, which cleaves serine- or threonine-linked oligosaccharide, showed no effect (results not shown). Together, these results indicate that the carbohydrates linked to the LPXTGase are essential for catalytic activity.

Amino Acid Composition of LPXTGase—Amino acid composition of the purified enzyme revealed 61 amino acid residues with an aggregate mass of 6,306 daltons (Table II). The enzyme contains only 11 amino acid species and no aromatic amino acid, which explains the failure of the enzyme to absorb UV at 280 nm and the failure to be stained by Coomassie Blue. Interestingly, the enzyme contains only a few hydrophobic amino acids but an unusually large number of alanines. A most striking feature is the presence of unusual (unknown) amino acids, with an aggregate mass of about 3,000 daltons, or about 30% of the enzyme's protein backbone. The unusual hydrophobicity of the enzyme appears to be imparted by these unknown amino acids, as will be discussed below.

Amino Acid Sequence of a Tryptic Fragment of the LPXTGase—Despite its purity, several attempts to determine the amino acid sequence of the enzyme using an automated sequencer were unsuccessful, even when efforts were made to sequence the deglycosylated core protein. Because this suggested that the N terminus might be blocked, we attempted to sequence an internal tryptic fragment of the core protein, also without success.

The sequence failure suggested either that the standard program used for automated sequencing could not be applied to this peptide or that the sequence was made up of unusual amino acids. Thus, we elected to sequence an internal fragment manually. For this purpose, the enzyme was first treated with *N*-glycosidase F and *O*-glycosidase, and the deglycosylated core protein was separated on silica gel TLC (Fig. 9*A*). When 80% ethanol was used as the running solvent, the core protein moved with an R_F of 0.6, which was closely followed by Hepes buffer, while the carbohydrate remained at the origin. Under identical TLC conditions, untreated enzyme, bovine serum albumin, ovalbumin, and trypsin remained at the origin (not shown). The high R_F value of the core protein verifies its high hydrophobicity. The core protein was then digested with trypsin, and the digestion products were incubated with phenylisothiocyanate in order to produce PTH-peptides as described under "Experimental Procedures." The reaction products were separated on a silica gel TLC plate using *n*-butyl alcohol/hexane/acetic acid/water (40:40:9:1) as the running solvent. As shown Fig. 9*B*, two PTH-peptides were detected. Very faint UV-absorbing material remained at the origin, which is attributable to PTH-trypsin. Under identical TLC conditions, PTH-bovine serum albumin and PTH-trypsin also remained at the origin. The mobility of these PTH-peptides in the highly nonpolar solvent further verified the unusual hydrophobicity of the peptides. The slow moving fragment was then subjected to Edman degradation, and seven PTH-derivatives were obtained as described under "Experimental Procedures." Using a C-18 column, both the first and the second PTH-derivatives were identified to be PTH-proline, and the seventh was identified as PTH-aspartic acid/asparagine. But the third, fourth, fifth, and sixth PTH-derivatives exhibited retention times far longer than those of any known PTH-derivatives. The masses of these PTH-derivatives were 537.0, 537.0, 212.1, and 288.0 Da respectively, which do not match with the total mass of known PTH-derivatives.

TABLE II
Amino acid composition of LPXTGase
The amino acid composition of LPXTGase was determined by the Rockefeller University Protein Chemistry Laboratory.

Amino acids	Number of residues
Asn/Asp	5
Gln/Glu	10
Ser	3
Thr	1
Gly	5
Ala	24
Pro	3
Val	1
Leu	1
Ile	1
Lys	7
Unknown	?

Because of their high total mass, the possibility that the third and the fourth amino acids were covalently linked to an unknown molecule was considered. Therefore, the third PTH-derivative was acid-hydrolyzed with 6 N HCl for 22 h at 110 °C, and the products were analyzed by a C-18 reverse phase column (Fig. 10*A*) and mass spectroscopy (Fig. 10*B*). Whereas the reverse phase column chromatography revealed a single PTH-

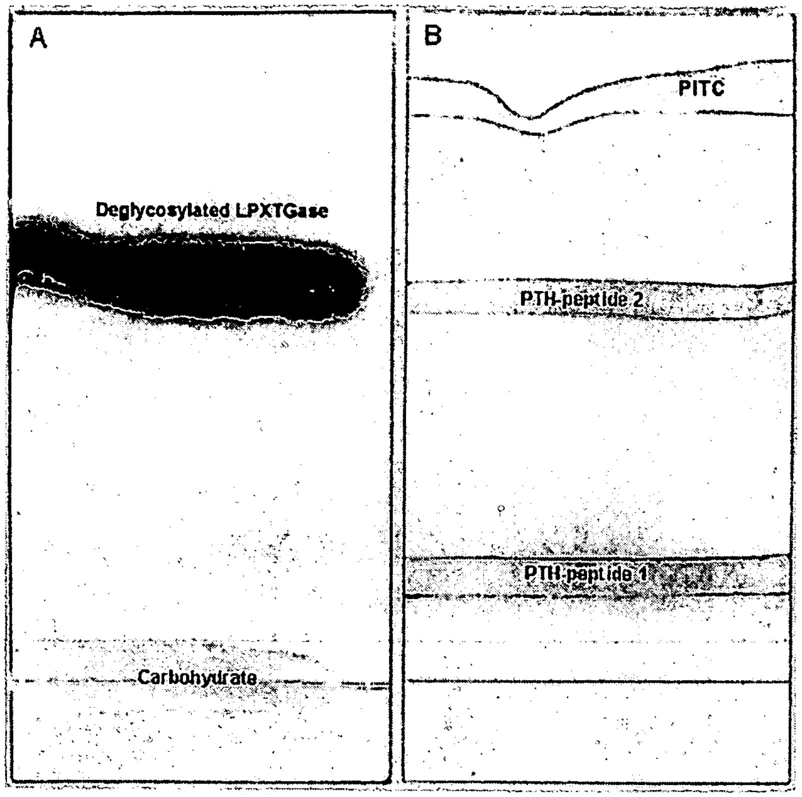

FIG. 9. **Purification of the core protein of LPXTGase and separation of the phenlthiohydantoin-labeled tryptic fragments of the core protein.** *A*, the LPXTGase was deglycosylated as described under "Experimental Procedures," and the core protein and carbohydrates were separated on silica gel TLC using 80% ethanol as a running solvent. *B*, the deglycosylated enzyme (core protein) was eluted from the plate and subjected to trypsin digestion, and the N termini of the tryptic fragments were linked to PTH as described under "Experimental Procedures." The PTH-labeled tryptic fragments were separated on silica gel TLC using a solvent mixture consisting of *n*-butyl alcohol, hexane, acetic acid, and water (40:40:9:1).

compound, with a retention time close to that for PTH-phenylalanine, the mass spectroscopy showed three distinct peaks with masses of 102.2, 288.1, and 519.2 Da, which do not correspond with those of known PTH-derivatives. Acid hydrolysis of the fourth PTH-derivative gave rise to identical results as the third PTH-derivative. Meanwhile, the acid hydrolysate of the fifth PTH-derivative contained two species with masses of 102.1 and 212.1, the latter representing unhydrolyzed material. Acid hydrolysate of the sixth PTH-derivative contained two species with masses of 102.1 and 288.0, the latter also representing the unhydrolyzed material. Thus, we could only conclude from the species generated by acid hydrolysis that the third, fourth, fifth, and sixth PTH-derivatives are not common amino acids.

DISCUSSION

The LPXTGase of *S. pyogenes* is an unusual enzyme in many respects. The enzyme is glycosylated, and the carbohydrate moiety appears to be essential for enzyme activity. This suggests that a certain spatial arrangement of carbohydrate and protein backbone is necessary for enzyme activity. As far as we know, no precedent for a glycosylated enzyme exists in prokaryotes. However, in eukaryotes, C1r and C1s, the subcomponents of the complement C1, and prekallikrein, a protease in the blood clotting cascade, are known to be glycosylated (62, 63). It is not known whether these carbohydrates play any role in the proteolytic reaction.

In the LPXTGase, the protein backbone is extremely hydrophobic, which may be attributed to the presence of uncommon amino acids. This is supported by the fact that the PTH-compounds of these unusual amino acids moved nearly to the solvent front with a running solvent mixture of *n*-butyl alcohol/hexane/acetic acid/water (40:40:9:1). Seven lysine residues were found in the enzyme, yet tryptic digestion of the enzyme yielded only two fragments, indicating that either several lysine residues were not accessible to trypsin or that some lysines were modified. It is tempting to speculate that in living bacteria, the hydrophobic protein backbone is embedded in the cell membrane and that the hydrophilic carbohydrate is localized within the hydrophilic peptidoglycan layer. The salt sensitivity of the LPXTGase activity probably reflects an intimate association of the enzyme with the hydrophobic environment of membrane.

Direct determination of the purity of the purified LPXTGase by standard procedures such as SDS-PAGE analysis was not possible because of its ability to form large aggregates in the presence of SDS, and as a result it is unable to enter the resolving gel as a distinct protein band and barely enters the 4% stacking gel. The protein does not contain any aromatic amino acids, which explains its inability to absorb UV at 280 nm, and does not stain with Coomassie Blue or silver. Even when over 200 μg of purified enzyme was subjected to SDS-PAGE, no protein band was detected after staining with Coomassie Blue or silver, indicating that the purified enzyme sample did not contain any stainable protein contaminant. We considered that the observed LPXTGase activity might be attributed to a very small amount of contaminant rather than the glycoprotein that we have identified. However, if this were true, we must conclude that the putative contaminant with endopeptidase activity is also a glycoprotein, because removal of sugars from purified endopeptidase preparations always abolished the enzyme activity.

Over the course of these studies, several independently purified LPXTGase samples were analyzed for amino acid sequence. In each analysis, 200 μg to over 1 mg dry weight of the enzyme sample was used. Since as little as a 0.1-μg sample of a 10-kDa peptide is sufficient for sequencing by the automated sequencer, 0.01–0.05% of contaminating proteins would have been detected by the instrument. In fact, 10 of our most puri-

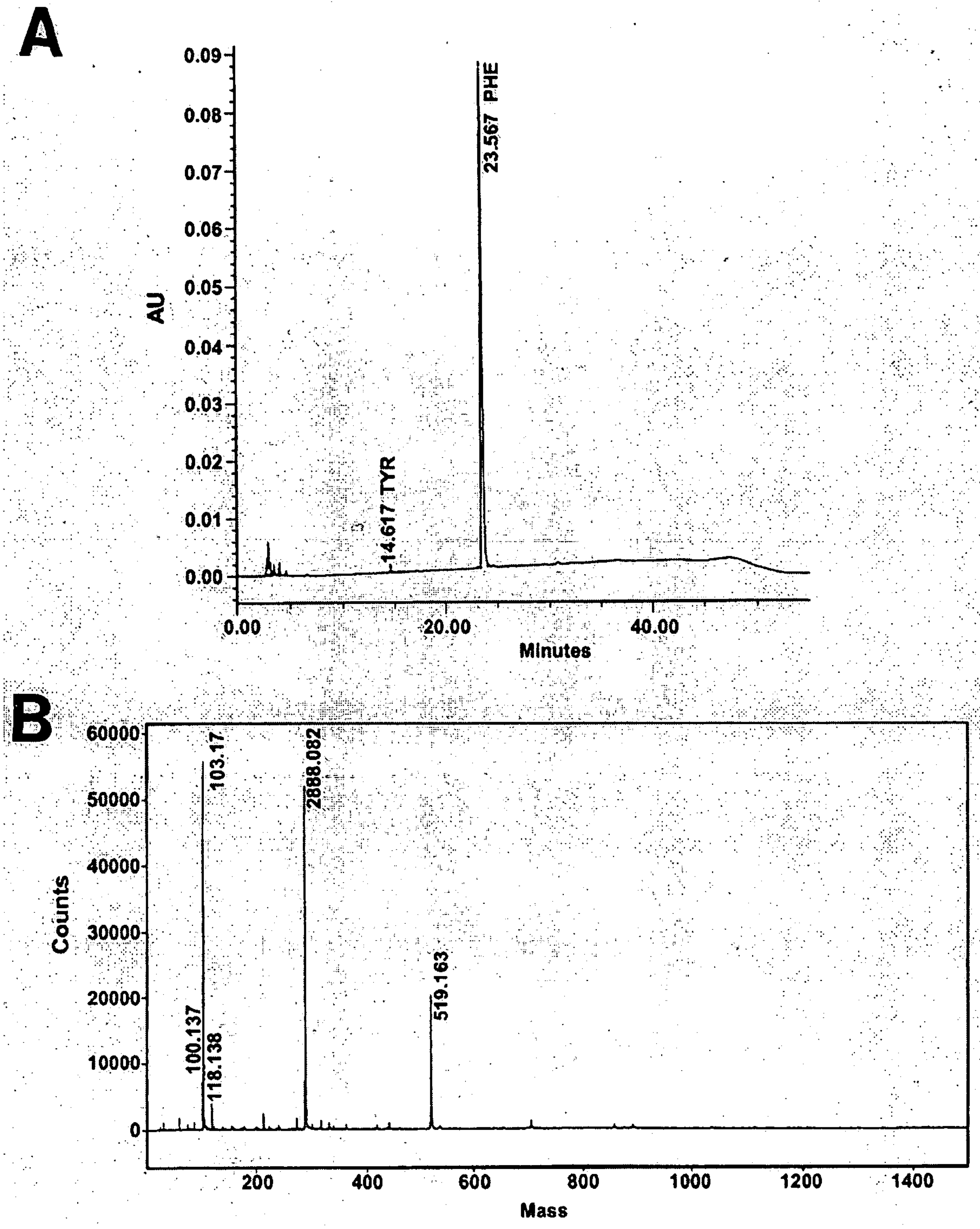

FIG. 10. **Analysis of an unusual amino acid present in peptide fragment 1.** The third PTH-derivative of tryptic fragment 1, exhibiting a mass of 537.0, was hydrolyzed for 22 h with 6 N HCl at 110 °C, and the hydrolysis products were examined with reverse phase chromatography (*A*) and mass spectroscopy (*B*).

fied samples could not be sequenced, and no false sequence data could be obtained, further verifying the purity of the preparation.

In contrast to the sortase described by Schneewind *et al.* (55), which cleaves the LPXTG sequence after threonine, our LPXTGase cleaves the bead-bound KRQLPSTGETANPFY after glutamic acid and cleaves the free form of the same pentadecapeptide after both serine and glutamic acid. In contrast to sortase, our LPXTGase does not have cysteine or methionine and is totally insensitive to sulfhydryl reagents. Another difference between LPXTGase and sortase is that hydroxylamine inhibits the activity of our LPXTGase, whereas hydroxylamine stimulates the activity of sortase. The LPXTGase activity was reduced by more than 70% in the presence of 100 mM hydroxylamine, and it was completely abolished in the presence of 200 mM.

Another clear distinction between sortase and LPXTGase lies in the substrate cleavage activities of the enzymes. The K_m value of 0.26 mM for LPXTGase for the KRQLPSTGETANPFY peptide was comparable with the reported K_m values of 0.21, 0.36–3.1, and 0.08–0.5 mM for human Lys-plasmin (64), bovine trypsin (65), and a protease from *S. aureus*, respectively (66), for synthetic peptides. The calculated turnover value, or K_{cat}, of the LPXTGase is 0.016/s. In comparison, the reported K_{cat} values for bovine trypsin ranged from 0.003 to 1.35/s, depending on the synthetic substrate used (65). When compared with the calculated K_{cat} of sortase from *S. aureus* (56), the 0.016/s K_{cat} value of LPXTGase from *S. pyogenes* is at least 2 orders of magnitude higher. It is estimated that there are at least five different surface proteins on a given Gram-positive bacterium that are anchored through the LPXTG motif, and there are probably thousands of copies of these molecules expressed in each organism. Thus, in order to properly anchor all these molecules during the 30–40-min division time of the bacterial cell, an enzyme with a high K_{cat} would be necessary to successfully accomplish this.

Many peptide antibiotics produced by *Bacillus* sp. contain unusual amino acids, and these peptides are synthesized nonribosomally on amino acid-activating polyenzyme templates. The overrepetition of alanine in the LPXTGase seems unusual; however, another characteristic of nonribosomally synthesized peptides is the overrepetition of some amino acids. For example, tyrocidine, a cyclic decapeptide antibiotic from *Bacillus brevis*, contains ornithine and three phenylalanines, two of which are in the D-configuration. The mechanism of nonribosomal peptide synthesis was most extensively investigated previously in the synthesis of tyrocidine (67–70). Recently, the genes encoding the polyenzymes of tyrocidine synthesis have been cloned (71).

It is surprising that *S. pyogenes* produces a small molecule that inhibits the activity of LPXTGase. We speculate that such an inhibitor may have some important regulatory function in the living bacterial cell. This is supported by the fact that *S. pyogenes* grown in the presence of this inhibitor fails to display M protein and fibronectin-binding protein on the cell surface,[2] suggesting that the function of this LPXTGase may be essential for cell surface expression of these proteins. Another important question is whether the LPXTGase and its inhibitor are present in other Gram-positive bacteria. When one assumes that surface proteins in most Gram-positive bacteria become anchored to cell wall peptidoglycan by a common mechanism, it is likely that the answer is yes. This has been verified from our recent finding that *S. aureus* also produces an enzyme strikingly similar to the LPXTGase of *S. pyogenes*. The *S. aureus* enzyme cleaves the LPXTG motif, has a similar molecular weight, and is glycosylated like the *S. pyogenes* enzyme.[3] In addition, we found a low molecular weight inhibitor from *S. aureus* that inhibits both the activity of this enzyme and the LPXTGase from *S. pyogenes*. An interesting possibility exists, therefore, that analogues of LPXTGase and sortase are present in all Gram-positive bacteria and that these two enzymes function together to accomplish the cleavage of the LPXTG motif of surface proteins and the ultimate anchoring of the cleaved protein to the cell wall. Precisely how these two enzymes accomplish this is currently being investigated.

Acknowledgments—We thank Peter Model and Emil Gotschlich for useful suggestions after reading the manuscript.

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[3] S. Lee and V. A. Fischetti, unpublished data.

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EXHIBIT C

Experiment 1: Determination of the Molecular Mass of the Staphylococcal LPXTGase Enzyme

Materials and Methods

Bacterial strain and culture. *S. aureus* strain RN4220 was grown in 25 liters of Todd-Hewitt medium supplemented with 1% yeast extract in a carboy with stirring by a motor-driven impeller. Cells were harvested when the optical density of the culture at 600 nm reached 1.2. To harvest the cells, the culture was concentrated to about 2 liters with a Millipore Procon filtration apparatus, and cells were then pelleted by centrifugation for 10 min at 8,000 rpm using a Sorvall GSA rotor. The cell pellet (about 80 g [wet weight]) was suspended in 1 liter of 20 mM Tris-HC1 buffer, pH 7.7, and the cells were pelleted again by centrifugation.

Cell lysis and preparation of crude extract. The washed cell pellet was suspended in 800 ml of 30 mM Tris-HCI buffer, pH 7.7, and then 3 mg of lysostaphin was added, and cell lysis was achieved by mixing the solution for 1 h at 37°C. Digestion of the cell wall peptidoglycan by lysostaphin resulted in cytosolic and membrane vesicle release. The cell lysate was then centrifuged for 20 min. at 10,000 rpm with a GSA rotor, and the supernatant containing cytosol and membrane vesicles was collected. The pellet, consisting of cell ghosts and unlysed cells, was suspended in 300 ml of Tris-HCI buffer, pH 7.7, containing 0.2% Brij 35, and the suspension was stirred overnight at 4OC. The suspension was then centrifuged for 20 min at 10,000 rpm, and the supernatant, termed membrane extract, was collected.

Purification of LPXTGase. To 800 ml of the cytosol fraction, Brij 35 was added to a final concentration of 0.1%. The cytosol fraction was then combined with the membrane extract (300 ml) and the mixture applied to a DEAE-cellulose column (20 by 5.1 cm), equilibrated with 20 mM Tris-HCI buffer, pH 6.8. After the mixture had entered the column, the column was eluted with 20 mM Tris-HCl buffer, pH 7.7, containing 0.1% Brij 35, until the W absorption reached baseline, requiring about 600 ml of the buffer. The fall-through fraction and the column wash were combined and concentrated to 20 ml using an Arnicon ultrafiltration apparatus fitted with a YM3 membrane with a 3-kDa size limit. One half of the concentrated enzyme solution was applied to a Sephadex G50 column (60 by 4.3 cm) equilibrated with 20 rnM Tris-HC1 buffer, pH 7.7, containing 0.1% Brij 35; the column was then eluted with the same buffer, and 25-ml fractions were collected. For the enzyme preparations used in amino acid and sugar composition analysis, the DEAE-cellulose chromatography and gel filtration steps were repeated once again.

Biochemical characterization. Enzyme concentration was determined using the BCA protein assay reagent, which measures peptide bonds.

Determination of molecular weight. (i) Gel filtration. Three hundred microliters of the concentrated LPXTGase (about 170 kg protein) was mixed with 200 µl of molecular weight standard proteins, and the mixture was applied to a Sephadex G50 column (48 by 2.4 cm) equilibrated with 20 mM Tris-HCI buffer, pH 7.7, containing 0.1 % Brij 35. The column was eluted with the same buffer solution, and 8-ml fractions were collected. The molecular markers were identified by UV absorption at 280 nm, and LPXTGase was identified by LPXTG peptide cleavage activity.

RESULTS

Molecular mass of S. *aureus* LPXTGase. Figure 1 shows the elution profiles of proteins of known molecular mass and S. *aureus* LPXTGase. From this, the molecular mass of LPXTGase was estimated to be 14 kDa , which is the same as the molecular mass of S. *pyogenes* LPXTGase, also estimated by gel filtration.

Experiment 2: Comparison of the Specificity of the Staphylococcus Enzyme with the Enzyme from Streptococcus

Material and Methods

Preparation of LPXTG peptide substrates. LPXTG-containing peptides, KRQLPSTGETANPF(Y) derived from the streptococcal M protein and AQA LPETGEENPF(Y) from staphylococcal protein A, were synthesized with a C terminal tyrosine by the Rockefeller University Protein DNA Technology Center. The terminal tyrosines were labeled with ^{125}I using Iodobeads. The labeled peptides were purified on silica gel TLC plates using propanol-pyridine-water (2:1:1) as a running solvent mixture. The N termini of the labeled peptides were then linked to carboxymethyl glass beads by carbodiimide catalysis according to the manufacturer's instructions and as described previously (Lee, S.G. *et al.* (2002), J. Biol. Chem. 277:46912-46922).

Enzyme assay method. Cleavage activity of the LPXTGase enzyme was determined using glass bead-bound KRQLPSTGETANPFY as a substrate, where the terminal tyrosine was labeled with ^{125}I, as described previously (Lee, S.G. *et al.* (2002), J. Biol. Chem. 277:46912-46922). Briefly, aliquots of 2 to 40 µl of enzyme were introduced into Microfuge tubes, after which 30 mM Tris-HCI buffer, pH 7.7, containing 0.1% Brij was added to achieve a 40-µl volume. A 10-µl water suspension of bead-bound, ^{125} I-labeled LPXTG peptide was added to the tubes, and the reaction mixtures were shaken at 37°C for 1 h. At the end of the reaction, 150 µl of distilled water was added to each tube and the tubes were vortexed. The tubes were then centrifuged, and a 150-µl aliquot of the supernatant was withdrawn and radioactivity counted. The release of cleaved, ^{125} I -labeled peptide fragment from the beads was the measure of enzyme activity. The tubes with all components except for the enzyme served as background control.

Identification of enzyme reaction products. About 12 µg of ^{125} I -labeled (1.4 X 10^6 cpm) AQALPETGEENPFY peptide in 50 µl of distilled water was added to two separate microcentrofuge tubes. To one tube was added -6 μg of *S. aureus* LPXTGase in 50 μl of 60 mM Tris-HC1 buffer, pH 8.0, containing 0.2% Brij, and to the second tube was added -6 μg of S. *pyogenes* LPXTGase in 50 μl of the same buffer solution. The reaction mixtures were shaken at 37°C, and at designated times 10-μl aliquots were removed and spotted onto silica gel TLC plates. The plates were developed with a solvent mixture consisting of ethylacetate-pyridine-acetic acid-water (60:30:9:24), and the reaction products were identified by autoradiography. Reaction products were eluted from the plates, and amino acid sequences were determined with an Applied Biosystems AB 1 Procise 498 instrument by the Protein/DNA Technology Center of the Rockefeller University. The ^{125}I-labeled KRQLPSTGETANPFY peptide, derived from streptococcal M protein, was cleaved by LPXTGase from both *S.pyogenes* and *S. aureus* to determine the cleavage location in the LPXTG motif of this peptide as described above.

Other materials. Silica gel thin-layer chromatography (TLC) plates (catalog no. M5729-6) and solvents were purchased from Fisher. Carboxymethyl glass beads (catalog no. G-3910), 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (catalog no. B-1911), and molecular weight markers blue dextran (catalog no. D-4772), carbonic anhydrase (catalog no. C-7025), cytochrome c (catalog no. C-7150), aprotinin (catalog no. A-3886), and vancomycin (catalog no. V-2001) were purchased from Sigma. Iodobeads (product no. 28665), fluorescein isothiocyanate (FITC) (catalog no. 461 lo), and bicinchoninic acid (BCA) protein assay reagent (product no. 23225) were purchased from Pierce.

RESULTS

Enzyme reaction product

We subjected the LPXTG-containing segment of the streptococcal M protein, KRQLPSTGETANPFY, to both S. *aureus* and *S. pyogenes* LPXTGases. As seen in Fig. 2, both enzymes cleave the streptococcal substrate after serine and glutamic acid, yielding two radioactive products, TGETANPFY and TANPFY.

Experiment 3: Enzyme Linked Sugars and Inactivation of the Staphylococcus Enzyme by Treating with Glycosidases

Materials and Methods

Analysis of enzyme-linked sugar species. About 220 μg of purified S. *aureus* LPXTGase in 0.5 ml Tris-HCI buffer was digested with 10 U of N-glycosidase F as described above. The digestion products were mixed with 50 ml of distilled water, and the mixture was subjected to ultrafiltration using an YM3 membrane with a 3,000-Da size limit. The filtrate was lyophilized, and the dried material was dissolved in 0.2 ml of distilled water. The concentrated filtrate was then applied to a P2 gel filtration column (48 by 1.4 cm) equilibrated with distilled water, the column was eluted with distilled water, and 3-ml fractions were collected. Aliquots of 0.3 ml from each fraction were transferred to microcentrofuge tubes and volumes reduced to 20 μl by means of a SpeedVac. The concentrated column fractions were spotted on a silica gel TLC plate, and sugars were detected as described above. The remainder of the column fractions was analyzed for sugar composition by the Complex Carbohydrate Research Center of the University of Georgia, Athens, Ga.

Enzymes. All enzymes used were purchased from Sigma. These were N-glycosidase F (catalog no. G-5166), β -glucosidase (catalog no. G-6906), β-galactosidase (catalog no. G-5635), β -N-acetylhexosaminidase (catalog no. A-7708), β -mannosidase (catalog no. M-9400), α-mannosidase (catalog no. M-7257), lysostaphin (catalog no. L-4402), and trypsin (catalog no. T-1426).

Inactivation of S. *aureus* LPXTGase by glycosidases. About 1 μg S. *aureus* LPXTGase in a 2-μl volume was added to six microcentrofuge tubes. To each of the first four tubes was added 0.1 U of β-glucosidase, β -mannosidase, α-mannoside, or N-acetylhexosaminidase in 8 μl of 20 mM Na-acetate buffer, pH 5.5, containing 0.1% Brij 35. To each of the next two microcentrofuge tubes containing LPXTGase was added 0.1 U of β -galactosidase or N-glycosidase F in 8 μl of 20 mM Tris-HCI buffer, pH 7.6, containing 0.1% Brij 35. The reaction mixtures were incubated at 37°C for 1 h. At the end of glycosidase treatment, 1 μl of 300 mM Tris-HCI buffer, pH 8.9, was added to the first four microcentrifuge tubes which contained acetate buffer, after which 30 μl of 30 mM Tris-HCI buffer, pH 8.0, containing 0.1% Brij was added to all microcentrifuge tubes. After adjusting the pH of reaction mixtures to about 8.0, about 1 μg of beadbound ^{125}I-labeled KRQLPSTGETANPFY (1.7 X 10^5 cpm) in 10 μl of distilled water was added to each tube, and the peptide cleavage reaction was carried out as described above for 1 h at 37°C.

RESULTS

Inactivation of S. *aureus* LPXTGase by glycosidases.

We had shown in our studies with the S. *pyogenes* enzyme that sugars are an integral part of S. *pyogenes* LPXTGase and certain sugars are essential for LPXTGase activity. The striking similarity of the LPXTGase enzymes from S. *aureus* and *S. pyogenes* in their chromatographic behavior and LPXTG peptide cleavage reactions prompted us to examine whether sugars are also essential for the *S. aureus* enzyme activity. *S. aureus* LPXTGase was therefore incubated with various glycosidases prior to testing for cleavage activity. As seen in Fig. 3, and similar to the case with S. *pyogenes* LPXTGase, pretreatment of the staphylococcal enzyme with N-glycosidase F β-glucosidase, α-mannosidase, β -mannosidase, or β -N-acetylhexosaminidase greatly reduced LPXTGase activity, but β -galactosidase did not. The possibility that protease contaminants in the commercial glycosidases might be responsible for the inactivation of LPXTGase was considered. We therefore incubated ^{125}I- labeled bovine serum albumin with the glycosidases that were used in the inactivation experiments. Only N-acetylhexosaminidase showed a trace amount of protease activity, while all other glycosidases showed no protease activity (results not shown).

Experiment 4: Reactivity of Staphylococcus Enzyme with Antibody Prepared Against the Streptococcus Enzyme

Material and Methods

Anti-LPXTGase antibody. The antibody against LPXTGase from S. *pyogenes* was produced in rabbits (Covance) by a primary dose of 200 μg of protein emulsified in complete Freund's adjuvant followed by two monthly doses of 100 μg/ml in incomplete adjuvant. Animals were bled 7 days after the last boost.

RESULTS

Reactivity of S. *aureus* LPXTGase with anti S. *pyogenes* LPXTGase antibody. About 4 μg (each) of S. *pyogenes* LPXTGase and *S. aureus* LPXTGase in *5* μl of 20 mM Tris buffer was spotted on a nitrocellulose sheet. The enzymes were then fourfold serially diluted in phosphate-buffered saline and spotted linearly on the sheet. The nitrocellulose sheet was then reacted with the *S. pyogenes* LPXTGase antibody, and after washing, the sheet was reacted with anti-rabbit antibody conjugated with alkaline phosphatase. After washing again, alkaline phosphatase activity was detected with 5-bromo-4-chloro-3-indolyl phosphate-Nitro Blue Tetrazolium as the substrate. Results are shown in Figure 4.

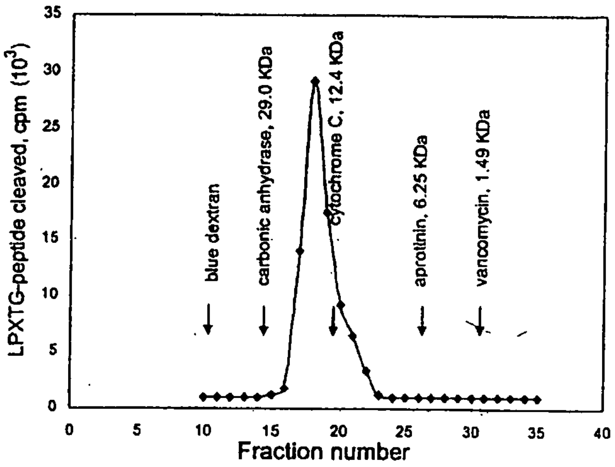

LPXTG-peptide cleaved, cpm (10³)
0
5
10
15
20
25
30
35
blue dextran
carbonic anhydrase, 29.0 KDa
cytochrome C, 12.4 KDa
aprotinin, 6.25 KDa
vancomycin, 1.49 KDa
0
5
10
15
20
25
30
35
40
Fraction number

FIGURE 1

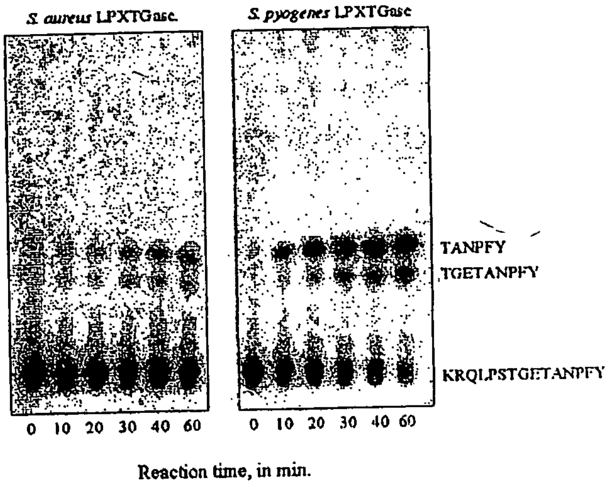
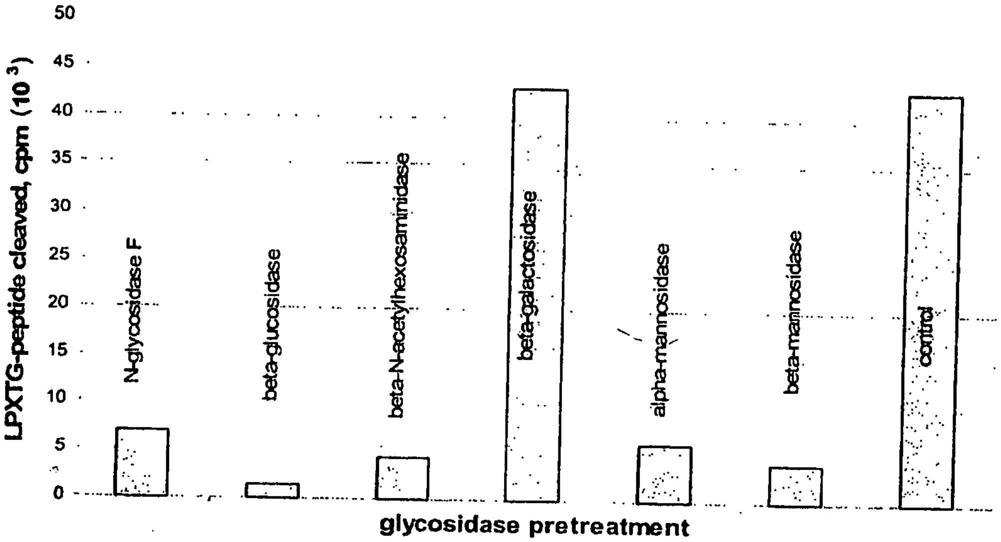

S. aureus LPXTGase
S. pyogenes LPXTGase
TANPFY
TGETANPFY
KRQLPSTGETANPFY
0 10 20 30 40 60
0 10 20 30 40 60
Reaction time, in min.

FIGURE 2



50
45
40
35
30
25
20
15
10
5
0
LPXTG-peptide cleaved, cpm (10³)
N-glycosidase F
beta-glucosidase
beta-N-acetylhexosaminidase
beta-galactosidase
alpha-mannosidase
beta-mannosidase
control
glycosidase pretreatment

FIGURE 3

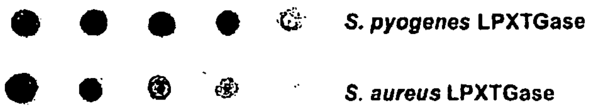

S. pyogenes LPXTGase
S. aureus LPXTGase

FIGURE 4